

# Technical Factors Affecting the Reproducibility of Intravesical Mouse Bladder Tumor Implantation during Therapy with *Bacillus Calmette-Guérin*<sup>1</sup>

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## ABSTRACT

Four methods of intravesical implantation of the transplantable mouse bladder tumor, MBT-2, and their effects on intravesical therapy with *Bacillus Calmette-Guérin* (BCG) were compared, and modifications which improved implantation are described. Pretreatment of the bladder with *N*-methyl-*N*-nitrosourea (MNU) resulted in tumor implantation in approximately two-thirds of the animals; however, all tumors penetrated the bladder wall. Using the MNU implantation procedure, intravesical BCG therapy was shown to reduce MBT-2 outgrowth by 77%. Tumor cell instillation after electrocautery produced an incidence of tumor implantation similar to that of the MNU procedure. The efficacy of BCG for the electrocautery implantation procedure also was similar to the MNU method. With the electrocautery procedure, the electrode and tumor cells were introduced into the bladder via a catheter prepared from PE 10 polyethylene tubing. The procedure required two catheterizations and produced a 24% incidence of extravesical tumors. Use of a Teflon catheter and a single catheterization for tumor cell instillation resulted in a reproducible method for implanting MBT-2 tumors which were all confined within the bladder. The efficacy of BCG therapy was unchanged from that described for the other implantation techniques.

## INTRODUCTION

In 1976, Morales *et al.* (9) first reported the effective use of intravesical BCG<sup>5</sup> in the treatment of superficial bladder cancer. Since this original observation, BCG has been used successfully in the treatment of carcinoma *in situ* (4), residual superficial bladder tumors (3, 10), and as a prophylactic agent in patients rendered tumor free by endoscopic resection (2, 6). Recent prospective, randomized clinical trials have shown clearly that BCG is one of the most effective means of treating patients with superficial bladder tumors (2, 6). While these studies have shown BCG to be an effective therapeutic agent, many questions remain to be answered including the preferred dose, treatment schedule, route of administration, appropriate BCG strain, the long-term toxicity of BCG therapy, and the mechanisms by which BCG mediates antitumor activity. Many of these questions can be effectively addressed in an animal model in which controlled experiments with large numbers of animals are performed.

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<sup>5</sup> The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; MNU, *N*-methyl-*N*-nitrosourea; PBS, 0.1 M phosphate-buffered saline; i.d., intradermally.

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The most appropriate model for the study of BCG therapy should include the natural anatomical site, *i.e.*, the bladder. Littman *et al.* (7) showed that the efficacy of BCG therapy for line 10 guinea pig hepatoma tumors was organ dependent. Intravesical injection of BCG for the treatment of implanted i.d. tumors was an effective means of therapy, whereas intravesical BCG therapy of tumors implanted i.m. was significantly less effective. Furthermore, Weinstock and Boros (16, 17) showed that the granulomatous inflammatory response was organ dependent. These investigators demonstrated that both the composition and functional activity of the infiltrating mononuclear cells varied among different organ sites.

We have adapted an intravesical tumor implantation model for the study of BCG therapy which was originally described by Soloway (13) as a means of screening chemotherapeutic drugs for efficacy in bladder cancer. This model uses a chemically induced transplantable bladder, MBT-2. Previous studies have shown that the MBT-2 tumor shares many characteristics with human transitional cell carcinoma including chemical etiology (14), histology (14), weak immunogenicity (8), and response to chemotherapeutic agents (14). Moreover, recent studies by us (12) and others (5, 11) have demonstrated that the response of MBT-2 tumors to BCG therapy correlates well with the observations reported in human clinical trials.

In this paper, we compare previously reported bladder implantation models (1, 13, 18) and describe our modifications which provide reproducible tumor implantation percentages and well-confined bladder tumors. In addition, we describe the effects of the different implantation procedures on the efficacy of intravesical BCG therapy.

## MATERIALS AND METHODS

### Animals

Six- to 8-week-old female C3H/He mice were used throughout the study. Animals were housed 10 to 20 mice/cage and fed Purina laboratory chow and water *ad libitum*.

### Preparation of Bladder Tumor for Implantation

The mouse bladder tumor MBT-2 (kindly provided by Dr. Mark Soloway, University of Tennessee Medical Center, Memphis, TN) was obtained from solid tumors growing *s.c.* in the flanks of C3H/He mice. The tumors were processed as described previously (12), and the appropriate viable cell number was instilled into the bladder in 0.1 ml of PBS, pH 7.2, containing 0.01 M phosphate ( $K_2HPO_4$  and  $NaH_2PO_4$ ) and 0.15 M NaCl.

### Anesthesia

For preimplantation treatment with MNU, ether inhalation anesthesia was used. Using ether anesthesia, the mice remained asleep for less than 5 min and voided soon after awakening, thus eliminating most of the instilled material. The cumulative mortality rate for ether anesthesia

was approximately 10%.

In the experiments in which bladder cautery was used, the anesthetic was sodium pentobarbital administered i.p. (0.05 mg/g body weight). This form of anesthesia was required to eliminate the hazard of using cautery in the presence of ether. An additional benefit of pentobarbital was that mice remained anesthetized longer and did not void for at least 30 min. The cumulative anesthesia-related death rate with pentobarbital was 2%.

**Models of Bladder Tumor Implantation**

**Method 1.** MNU (0.1 ml) at a concentration of 20 mg/ml in 0.15 M sodium acetate buffer was instilled into 53 ether-anesthetized mice. The instillation of MNU was performed transurethraly through a PE 10 polyethylene catheter (Fig. 1A) using a  $\times 16$  magnification by a Zeiss operating microscope as originally described by Weldon and Soloway (18). Twenty-four hr later, MBT-2 cells were instilled via PE 10 catheter. BCG therapy (160 mg, Pasteur strain, in 0.1 ml PBS) was initiated 24 hr after tumor cell instillation. Mice were treated weekly for 4 weeks. Control mice were treated with diluent only during the therapy protocol.

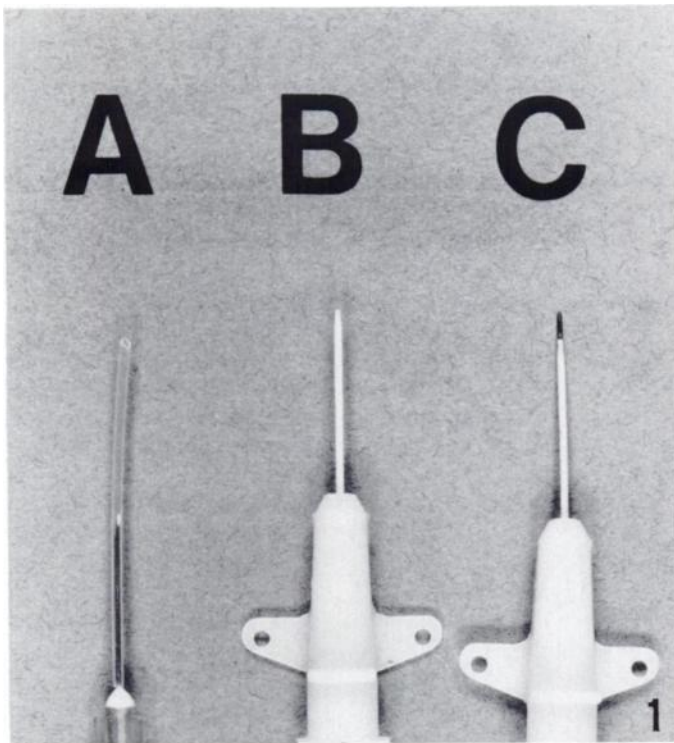


Fig. 1. Catheters used for tumor implantation procedures. A, PE 10 polyethylene tubing; B, Teflon catheter; C, Teflon catheter with cautery electrode inserted.

**Method 2.** Mice were placed under light general anesthesia with i.p. injection of sodium pentobarbital (0.05 mg/g animal weight) and secured on a grounded plate with an electrical conducting gel between the shaved skin and the plate. A PE 10 catheter (Fig. 1A) was introduced into the bladder, and a stainless steel wire electrode was inserted through the catheter. The electrode protruded 1 mm outside the PE 10 catheter (as shown for the Teflon catheter, Fig. 1C). The length of the catheter was determined in separate experiments in which mice underwent laparotomy to confirm the position of the catheter and electrode. The electrode was activated with a Bovie unit for 4 sec at the lowest coagulation setting, and the catheter and electrode were removed to enable instillation of tumor cells through a second PE 10 catheter. BCG (160 mg, Pasteur strain, in 0.1 ml PBS) therapy was initiated 24 hr after tumor instillation. Mice were treated weekly for 4 weeks. Control mice were treated with diluent only during the therapy protocol.

**Method 3.** The same procedure as described for Method 2 was used

except that a 24 G Teflon catheter (Fig. 1B) replaced the PE 10 catheter. The Teflon catheter was obtained from Travenol Laboratories, Inc. (Deerfield, IL) and is 1.6 cm long with a Leur plug. Catheterization with the Teflon catheter was smoother, and injuries to the urethra and bladder neck were reduced. The entire length of this catheter was introduced with no pressure on the pubic bone. Using this procedure, the fundus of the bladder was cauterized without perforation. Again, the appropriate catheter and electrode lengths were established in laparotomized mice. We have used this procedure successfully on  $20 \pm 3$ -g mice of the following strains: C3H/He; C57BL/6; C57BL/10; and A/J.

**Method 4.** The mice were treated in a manner identical to Method 3 except that only one catheterization was used. In this procedure, the electrode was removed from the catheter after cautery, and MBT-2 tumor cells were introduced through the same catheter. The single catheterization further reduced injuries to the urethra and bladder neck.

**Dose Response of Tumor Implantation**

In the dose response study, we instilled varying concentrations of viable MBT-2 tumor cells to determine the minimum number of cells required to produce tumors in a majority of the mice. The tumor cell concentrations used were  $2.5 \times 10^5$ ,  $5 \times 10^5$ ,  $7.5 \times 10^5$ , and  $10^6$  viable cells per mouse.

**Assessment of Tumors**

The mice were sacrificed after the fifth treatment (4 weeks) or when large tumors were palpated during treatment. All the mice underwent exploratory laparotomy to determine tumor location, i.e., whether confined inside the bladder or extending beyond the bladder wall. Metastases of the MBT-2 were not observed in any experiment. The term "extravesical tumor" denotes tumors not confined to the bladder because of excessive damage to the bladder wall during the instillation procedure. Fig. 2 demonstrates the types of tumors observed: Bladder 1, normal; Bladder 2, BCG treated; Bladder 3, tumor confined within bladder; Bladder 4, extravesical tumor.

All bladders and tumors were removed, and in selected cases, histology of the tumor was confirmed by hematoxylin-eosin staining.

**RESULTS**

**Method 1.** In Method 1 (MNU plus 2 PE 10 catheterizations) 53 mice were tested. MBT-2 tumor cells were implanted in all

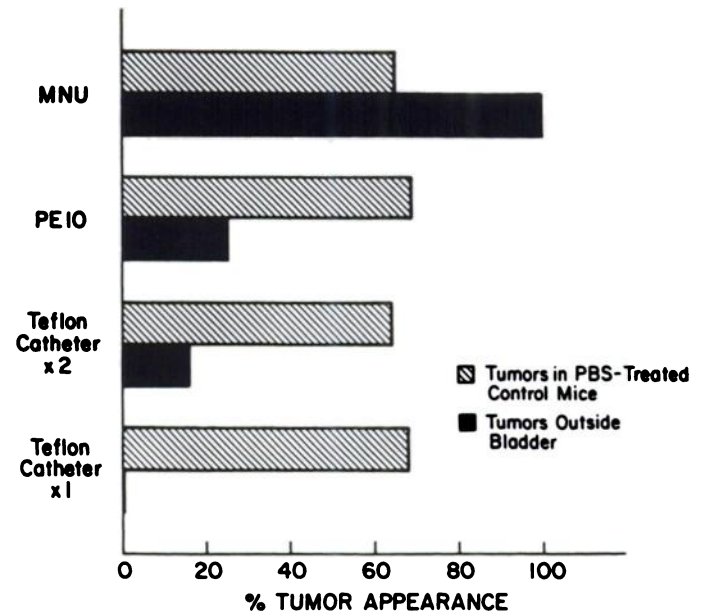


Chart 1. Effect of tumor implantation procedure on the outgrowth of MBT-2 tumors in PBS-treated control mice. All mice underwent cautery, tumor cell instillation, and weekly treatment with PBS as described in "Materials and Methods."

mice. Twenty mice received intravesical PBS, and 33 received intravesical BCG. In the PBS-treated control group, 13 of 20 (65%) developed tumors, all of which were extravascular tumors (Chart 1). In the BCG-treated group, 5 of 33 mice (15%) developed tumors (Chart 2). Also, in this group no tumor was confined to the bladder.

**Method 2.** In Method 2 (*cautery plus 2 PE 10 catheterizations*), 148 mice were treated with either PBS or BCG after separate PE 10 catheterizations for cautery and tumor implantation. One-hundred-fifteen mice were treated with PBS, of which 79 (69%) developed tumors (Chart 1). The appearance of extravascular tumors was reduced to 24%. The remaining 33 mice were treated with BCG (Chart 2). Only 5 of these mice (15%) developed tumors; no tumors penetrated the bladder.

**Method 3.** In Method 3 (*cautery plus two 24G Teflon catheterizations, once for cautery and once for tumor instillation*), 31 mice were tested. Eleven mice were treated with PBS, of which 7 (64%) developed tumors (Chart 1). Only 14% (one of 7) were extravascular tumors. The remaining 20 mice were treated with BCG. Only 5 (25%) developed tumors, and of these, none was extravascular (Chart 2).

**Method 4.** In Method 4 (using a single Teflon catheterization for cautery and tumor cell instillation), 95 mice were tested. Twenty-eight were treated with PBS, and 19 (68%) developed tumors (Chart 1). All tumors were confined within the bladder. The remaining 67 mice were treated with BCG. Only 17 mice

(25%) developed tumors, and all were confined within the bladder (Chart 2).

**Dose Response of Tumor Implantation.** The results of the dose-response studies of tumor implantation are shown in Chart 3. The lowest tumor cell inoculum that resulted in tumor outgrowth in the majority of the mice was  $5 \times 10^5$ . The same tumor cell dose provided equivalent rates of tumor implantation without regard to the method of bladder injury used prior to tumor cell instillation.

**DISCUSSION**

Recent prospective, randomized clinical trials have shown that intravesical BCG is an effective means of treating superficial bladder tumors (2-4, 6, 9, 10). These studies have demonstrated clearly the efficacy of BCG against bladder cancer, but numerous questions remain, which can be most easily addressed by the development of a reproducible animal bladder tumor model.

Soloway (13) introduced the intravesical implantation of the chemically induced bladder tumor, MBT-2, following severe denudation and ulceration of the bladder mucosa by administration of MNU prior to tumor instillation. Our experience using MNU revealed major problems with this method. First, after MNU instillation, all implanted tumors were extravascular. Whether this was a result of MNU alone or a combination of MNU and damage from the PE 10 catheterizations is not clear. The combined use of cautery and PE 10 catheterization reduced the extravascular involvement to 24%, suggesting that MNU was a primary cause of extravascular tumors. Also, the MNU protocol required that mice be anesthetized on 3 consecutive days during the first week of therapy. As a result, anesthesia-related mortality was unacceptably high (approximately 10%). Finally, MNU is unstable in solution, which introduces further technical problems and added expense.

Tumor implantation following cautery also was initially described by Soloway and Masters (15). These investigators suggested that cautery more closely mimicked the events occurring during transurethral resection of bladder tumors in that injuries to the mucosa by the cautery may allow implantation of tumor cells. In the mouse model, Soloway and Masters (15) used a PE 10 polyethylene catheter to introduce the cautery electrode and for instillation of tumor cells into the bladder. The PE 10 catheter must be prepared by the investigator by cutting the desired catheter length from a roll of tubing. Polyethylene is hard and produces sharp edges when cut (see Fig. 1A), which increases injuries to the urethra and bladder during the catheterization procedure. These injuries increase the incidence of extravascular tumors. Using PE 10 catheters, Soloway and coworkers (15, 18) reported tumor appearance in diluent-treated control mice ranging from 54 to 91%. There was no mention made of the incidence of extravascular tumors.

Using the methods of Soloway and coworkers (15, 18) (Method 2), we found that 69% of the PBS-treated control mice developed tumors, and of these, 24% had extravascular tumors. Further improvement in the procedure was achieved by replacing the PE 10 catheter with a Teflon catheter. The Teflon catheter has a uniform rounded tip, which reduces tissue injury during catheterization (Fig. 1B). Simply by changing from a PE 10 catheter to the Teflon catheter, the incidence of extravascular tumors in control mice was reduced to 16%. When the number of catheterizations was reduced to one for both cautery and tumor instillation, the incidence of extravascular tumors was virtually

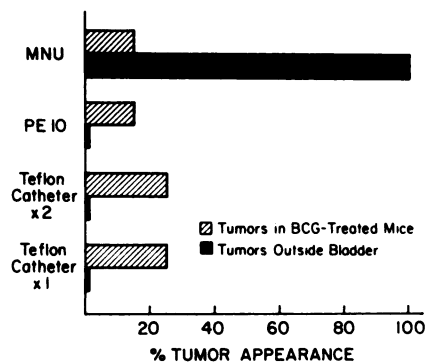


Chart 2. Effect of tumor implantation procedure on the outgrowth of MBT-2 tumors in BCG-treated mice.

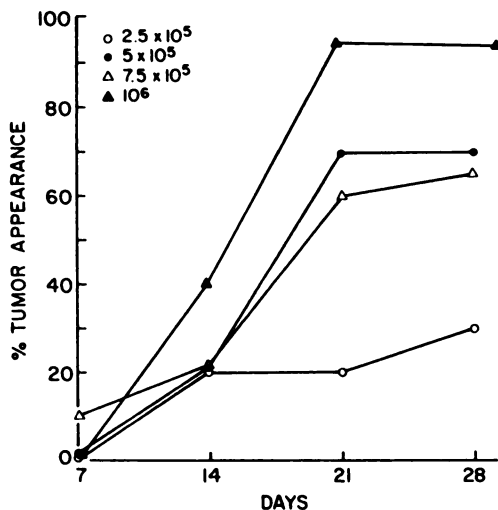


Chart 3. Effect of the number of viable MBT-2 tumor cells instilled into bladders on the percentage of tumor appearance.

eliminated. This final technical modification resulted in an animal model that more closely mimics human superficial bladder cancer in that the tumor is localized within the bladder. It should be stressed that the Teflon catheter (length, 1.6 cm) should be fully inserted into the bladder, aiming at the posterior wall with no pressure applied. This prevents penetration of the bladder by the electrode and extravascular tumor cell implantation.

Another important factor for obtaining reproducible tumor implantation results is the preparation of single cell suspensions of MBT-2 tumors. Regardless of the bladder injury procedure, we obtained a uniform percentage of tumor appearance in PBS-treated control mice (69, 64, and 68%). The uniform implantation results were achieved by processing the tumor for 20 min in trypsin (0.25% trypsin with 2 g EDTA/liter) which yielded approximately  $10^8$  cells/g of tumor with viability of greater than 90%. Under these conditions, implantation of  $5 \times 10^5$  viable MBT-2 cells resulted in development of tumors in about 65% of the mice. If viability of the single cell suspension was less than 90%, the percentage of mice that developed tumors also decreased (data not shown). Interestingly, the efficacy of BCG therapy was not altered by the method of tumor cell implantation (Chart 2). This was true even when 100% of the tumors were extravascular tumors as shown for Method 1 (MNU). These results suggest that the efficacy of BCG therapy may not be limited to superficial disease but also may be effective in the treatment of more invasive forms of bladder tumors. In this regard, preliminary studies show that BCG treatment of MBT-2 tumors implanted in bladders induces resistance to a second challenge at a distant site. Such resistance was not observed in mice treated with PBS or those receiving intravesical BCG in the absence of tumor instillation.

In conclusion, the tumor implantation procedures described herein provide reproducible implantation of MBT-2 tumors that are confined within the bladder. This implantation model provides a means of studying BCG therapy of superficial bladder tumors in the natural anatomical site.

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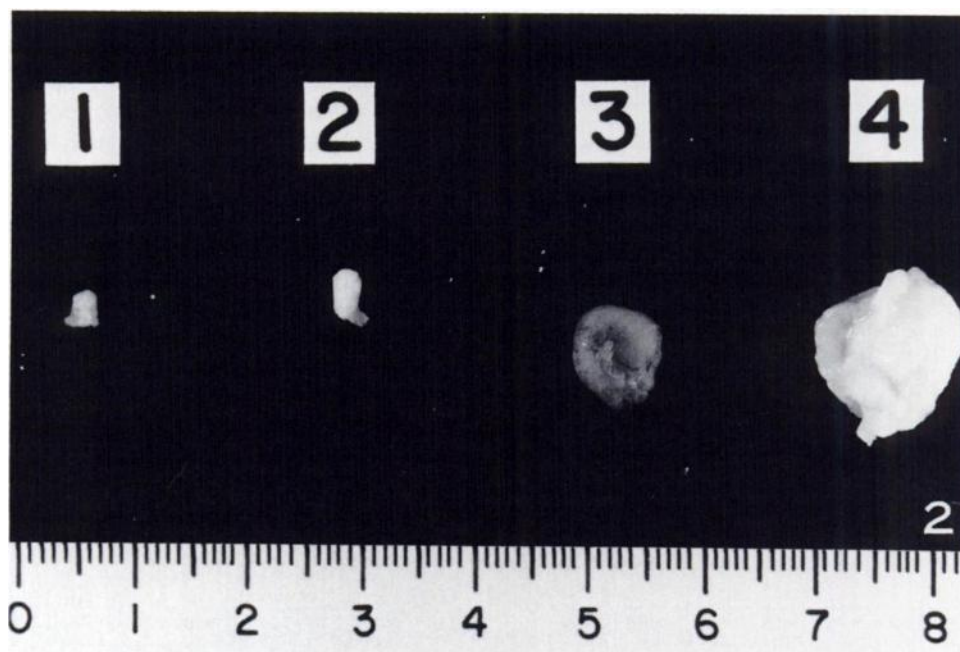


Fig. 2. Bladders from mice bearing implanted MBT-2 tumors. 1, normal bladder; 2, bladder with no tumor from BCG-treated mouse; 3, MBT-2 tumor confined to the bladder; 4, extravascular tumor.